thermoscientific

Detecting Stable Isotope Labeled Metabolites in Untargeted Analysis Using a Benchtop Orbitrap Mass Spectrometer and a Single Processing Software Platform

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ABSTRACT

Purpose: Develop an integrated mass spectrometry-based workflow for stable isotope labeling (SIL) in untargeted metabolomics covering data acquisition and analysis in a single experiment.

Methods: Metabolites from *E. coli* extracts consisting of unlabeled, ^{13}C-labeled and mixtures rendering incorporation levels of 10%, 25%, and 50% were analyzed with reversed phase separation and a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer. SIL data were processed using an untargeted approach including detection of labeled metabolites and unknown annotation.

Results: Untargeted metabolomics was applied to an SIL experiment. Confident formulas were generated using higher resolution settings along with accurate mass measurements enabling the detection of ^{13}C-incorporated isotopologues in labeled samples. Annotation capability facilitated detection of unexpected metabolites with label incorporation.

INTRODUCTION

SIL in metabolomics is a notable tool to elucidate metabolic pathway associations (tracer analysis) and rate of change of metabolites (flux analysis). Analytical methods employing SIL typically focus on defined pathways of interest employing a targeted approach, yet the interconnected network of metabolic pathways may involve unappreciated pathways. Further, SIL is used to increase confidence in unknown identification and to determine true sample related mass spectral features in untargeted analysis. As the role of SIL in untargeted metabolomics expands, the development of a facile, integrated workflow to support data acquisition and processing is needed. Here we developed an untargeted metabolomics approach applied to ^{13}C-labeled *E. coli* extracts using intelligent data-dependent acquisition and a single processing software platform.

MATERIALS AND METHODS

Sample Preparation

Dried down *E. coli* cell extracts purchased from Cambridge Isotope Laboratories were reconstituted in water containing 0.1% formic acid. Unlabeled and ^{13}C-labeled extracts were combined at defined ratios to obtain samples including 0%, 10%, 25%, 50% of ^{13}C-labeled metabolites.

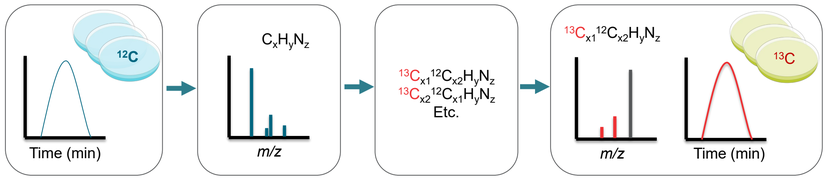
Liquid Chromatography-Mass Spectrometry

Metabolite separation was achieved with a Thermo Scientific™ Hypersil GOLD™ column (1.9µm, 150 x 2.1mm) on a Thermo Scientific™ Vanquish™ UHPLC system. Initial conditions consisted of 100% mobile phase A (water with 0.1% formic acid) at 300 µL/min, column temperature of 45° C and autosampler set at 5 ° C. Gradient elution was carried out for 8 min to 50% mobile phase B (acetonitrile with 0.1% formic acid) followed by a 1 min ramp up to 98% B and a 4 min isocratic hold. Total run time was 15 min with an injection volume of 2 µL. An Orbitrap Exploris™ 240 MS generated full scan data in positive mode with resolution settings at 30K, 60K, and 120K on all samples. A scan range of *m/z* 67 – 1000 enabled unbiased data collection. The S-lens was set to 70% RF and Thermo Scientific™ Easy-IC enabled internal calibration. Fragmentation spectra were collected using Thermo Scientific™ AcquireX intelligent acquisition on unlabeled sample at 30K resolution. HCD was performed with stepped collision energies of 30, 50 and 150%.

Data Analysis

A processing template for SIL in the Thermo Scientific™ Compound Discoverer™ software allowed data analysis for SIL incorporation (Figure 1), unknown annotation, and pathway mapping. Unknown annotation sources included ChemSpider for database searching and the online mzCloud™ library for MS/MS spectral matching.

Figure 1. Workflow to detect labeled compounds in Compound Discoverer software.



RESULTS

Figure 2. Detection of isotope pattern and fine structure were achieved with very high resolution. Combined with ≤1 ppm accurate mass measurements, confident elemental compositions are determined for the molecular species. Adenosine is putatively assigned with [M+H]$^+$ *m/z* 268.10385 and formula $C_{10}H_{13}N_5O_4$.

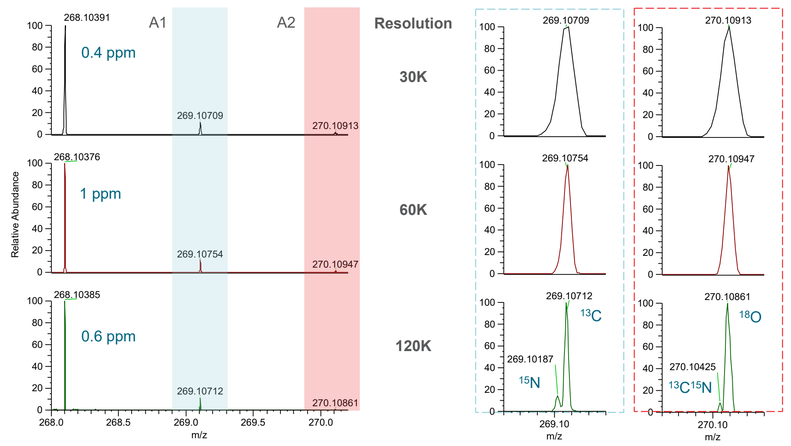


Figure 3. Detection of isotopologues and associated ions of labeled compounds resulting from formula. A) Chromatogram overlay of Tryptophan in unlabeled and labeled sample. B) Formula generated from isotope fine structure with 120K resolution and accurate mass resulting in $C_{11}H_{12}N_2O_2$. C) Mass spectrum of 50% ratio mix for Tryptophan. Ion detection includes the molecular ion, isotopologues and associated adducts.

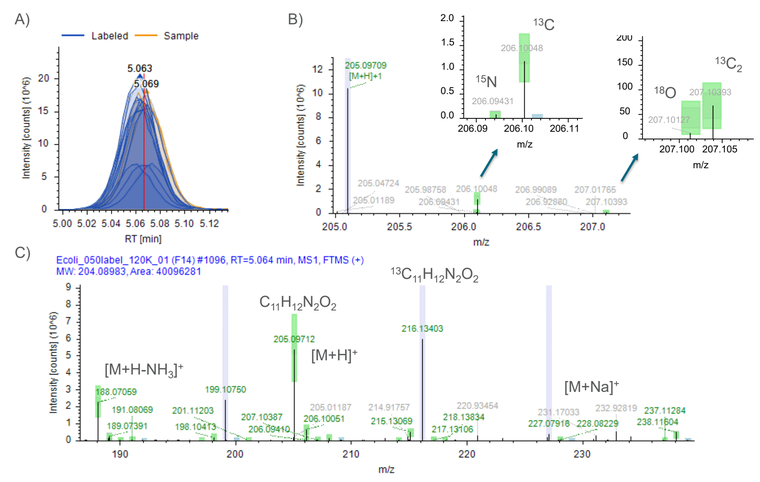


Figure 4. To assess the level of ^{13}C present, multiple mixtures containing different ratios, 10%, 25%, and 50% were compared to unlabeled sample. Annotated uracil, $C_4H_4N_2O_2$, confirms detection of ^{13}C-isotopologues at the expected levels corresponding to the defined ratios.

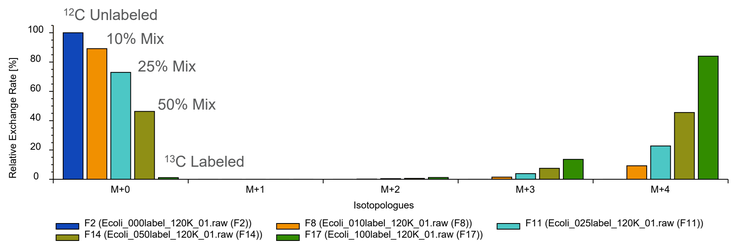


Figure 5. Reproducibility of isotopologue determination at different levels of ^{13}C incorporation. A) Uracil contains four carbons with a potential of five isotopologues. B) Relative exchange is computed for each replicate injection while displaying incorporation for individual isotopologues. Low %CVs were observed for all ratio mixtures.

A)

Uracil	^{13}C-Isotopologue
M+0	$C_4H_4N_2O_2$
M+1	$^{13}CC_3H_4N_2O_2$
M+2	$^{13}C_2C_2H_4N_2O_2$
M+3	$^{13}C_3CH_4N_2O_2$
M+4	$^{13}C_4H_4N_2O_2$

B)

Exchange Rate [%]					Rel. Exchange [%]	Triplicate Injections	%CV
0	1	2	3	4			
100	0	0	0	0	0	0% Label Incorporation	
100	0	0	0	0	0		
100	0	0	0	0	0		
89	0	0	1	9	10	10% Label Incorporation	5.4%
89	0	0	1	10	11		
89	0	0	1	10	11		
73	0	0	4	23	26	25% Label Incorporation	1.2%
74	0	0	4	22	25		
73	0	0	4	23	26		
46	0	1	7	46	51	50% Label Incorporation	1.1%
47	0	1	7	45	50		
47	0	1	8	45	51		
1	0	1	14	84	95	100% Label Incorporation	
2	0	1	14	83	94		
1	0	1	14	84	95		

Figure 6. An untargeted approach with SIL allows the detection of unexpected metabolites. Cytosine and Uracil are detected in the pyrimidine nucleobase salvage pathway using Metabolika pathways. The relative exchange is overlaid for both detected metabolites in this pathway.

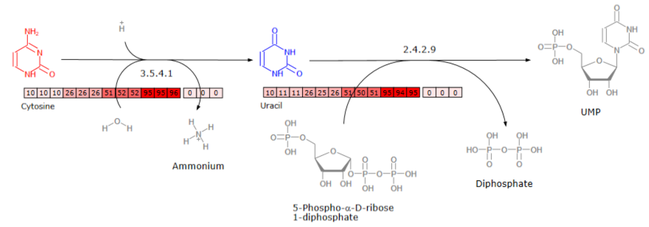
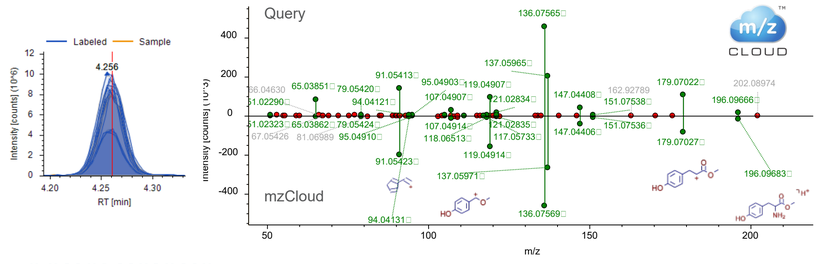


Table 7. Unknown annotation capability enables the detection of ^{13}C incorporation for unexpected compounds. Formula prediction and MS/MS spectral matching confirm the detection of putative tyrosine methyl ester in labeled samples.



CONCLUSIONS

A workflow to integrate untargeted metabolomics and SIL in a single experiment was developed.

- Higher resolution settings and accurate mass measurements generated confident formula determination leading to the detection of ^{13}C-incorporated isotopologues at defined levels.
- Implementing untargeted acquisition to obtain fragmentation spectra and utilizing annotation tools allowed for the confident detection of unexpected compounds with ^{13}C incorporation.

TRADEMARKS/LICENSING

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